

# Recovery Plan

*Cowpea mild mottle virus*  
*Carlavirus: Betaflexiviridae; order Tymovirales*

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December 15, 2014  
(Revised December 20, 2017)

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This recovery plan is one of several disease-specific documents produced as part of the National Plant Disease Recovery System (NPDRS) called for in Homeland Security Presidential Directive Number 9 (HSPD-9). The purpose of the NPDRS is to insure that the tools, infrastructure, communication networks, and capacity required to mitigate the impact of high consequence plant disease outbreaks are such that a reasonable level of crop production is maintained.

Each disease-specific plan is intended to provide a brief primer on the disease, assess the status of critical recovery components, and identify disease management research, extension and education needs. These documents are not intended to be stand-alone documents that address all of the many and varied aspects of plant disease outbreak and all of the decisions that must be made and actions taken to achieve effective response and recovery. They are, however, documents that will help USDA guide further efforts directed toward plant disease recovery.

## **Executive Summary**

Legume crops such as common bean (fresh and dry), cowpea, garbanzo bean (chickpeas), and peanut account for variable, but substantial acreages of legumes devoted to commercial (traditional and organic) production in many US states (<http://www.nass.usda.gov/>). Among legume crops in the U.S. soybean production occupies the greatest amount of land, at about 75,000,000 acres, valued at approximately 37 billion dollars in 2012) (<http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/related-data-statistics.aspx>).

This report addresses the exotic seed borne virus, *Cowpea mild mottle virus* (CPMMV) (genus, *Carlavirus*; family, *Betaflexiviridae*; order *Tymovirales*) that infects a wide range of cultivated legumes and may pose a threat to US legume production. Because of the magnitude and importance of soybean production to the US, aspects of this report address the possible threat of virus seed transmission to soybean, however, seed transmission by other legumes is also of great concern. This is because not only can the virus cause damage to the primary-infected legume species when it is seedborne, but it has potential to spread secondarily to other species by the whitefly vector.

The virus causes mild to severe mosaic and/or necrotic symptoms in leaves, stems, and pods in bean (*Phaseolus* species), cowpea (*Vigna* species), and soybean (*Glycine max*) varieties grown in the Americas, however, the extent of severity appears dependent upon the particular cultivar (possibly, genotype lineage). CPMMV is believed to have been introduced from western Africa where it is presumed to be endemic, into South America, from where it has putatively spread into the Caribbean region.

Initially reported to infect cowpea in east and then west African countries the virus spread largely unnoticed to India and southeast Asia, and most recently, to the Americas and Caribbean region. The first detection in the Americas was in soybean crops in Brazil and Argentina where losses have ranged from 10-100%. In particular, the recent outbreaks of stem necrosis of soybean and the re-emergence of CPMMV in common bean, have increased the level of concern about the virus and its potential future impact on susceptible genotypes grown in the Americas. The virus has subsequently spread to Puerto Rico, and possibly also to Mexico, on infected seed from South America and perhaps Africa as well. In Puerto Rico soybean and bean seed are routinely planted in winter nurseries for legume breeding and commercial seed production efforts. In the U.S. mainland, an isolate has been detected using

Next-Generation Sequencing (Illumina) in DNA extracts of the whitefly vector *Bemisia tabaci* (Genn.) in Florida 2014.

The virus is transmitted in a non-persistent manner by members of the whitefly sibling species group *B. tabaci* (Genn.), and importantly, through the seed of some legumes. Experimentally, CPMMV is readily mechanically transmissible through plant sap. The virus has an extremely broad host range among leguminous and non-leguminous hosts.

Currently, no monitoring is carried out to study the potential for, or to detect actual, introductions of CPMMV into the USA. Because it has not been found yet in the US mainland, no specific expertise exists to detect the virus visually, and no molecular detection assays are available. To avoid a disastrous introduction and subsequent spread, there is a need to develop training materials, and to train diagnosticians and legume growers who may encounter this disease on beans, cowpea, and soybean in particular.

Also, efforts should be pursued to fully characterize the strain(s) occurring in Puerto Rico, and more recently, in Florida, and elsewhere (potentially) in legume growing areas of the USA, particularly where the whitefly vector prevails. If introduced into the U.S. mainland, CPMMV has great potential to spread through seed, on infected ornamental or vegetable transplants, and by the viruliferous whitefly, itself if previously associated with a virus-infected host. Further, nursery and vegetable seedlings destined for commercial and home gardener markets are now frequently produced in states far from where they are eventually planted, and so this route of spread could be quite significant, particularly when plants are transported within and between states in mild climatic conditions that produce substantial acreages of susceptible legume species, including common bean, cowpea, garbanzo bean, and peanut, and in some instances, soybean.

The introduction of the virus, which is most likely to occur through the accidental importation of infected seed from already infected locales, could pose a high risk to legume crop species. Thus, phytosanitary measures are recommended to avoid the introduction and spread throughout the US mainland, and for eradication in Puerto Rico, where it already occurs in winter soybean nurseries, and perhaps elsewhere by now. The distribution and incidence of CPMMV at the time of detection would guide response recommendations, which may include quarantine and/or eradication programs aimed at preventing further spread of the viral pathogen.

***Cowpea mild mottle virus***  
***genus, Carlavirus; family, Betaflexiviridae; order Tymovirales***

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## I. Introduction

**First reports and economic damage.** Initial and subsequent reports of the extent of damage and economic importance of CPMMV to legume crops have been inconsistent. For example, the virus was reported to have little effect on the growth and yield of infected cowpea crops in Ghana (Brunt & Kenten, 1973), Nigeria (Anno-Nyako, 1987), and Papua New Guinea (Philemon, 1987), of mung bean and French bean in Tanzania (Mink and Keswani, 1987), and of French bean and soybean in Brazil (Costa et al., 1983). In contrast, CPPMV was responsible for 64-80% yield loss in groundnut (peanut) in Kenya (Bock et al., 1975, 1977). Elsewhere, in addition to annual losses in legume production resulting from CPMMV infection throughout western and eastern Africa, crop damage has been associated with whitefly outbreaks in Indonesia, resulting in 11-56% crop loss (Akin 2003; Time et al. 2010; Taiwo, 2003).

**The causal virus.** *Cowpea mild mottle virus* (CPMMV) is a single-stranded RNA virus encapsidated in a slightly curved, or 'flexuous' filamentous particle of 650 nm in size (Figure 1) that resembles those associated with members of the genus, *Carlavirus*. The virus forms inclusion bodies having a feather-like appearance in infected cells that differs from inclusion bodies observed for aphid-transmitted viruses (Figure 2) (Brunt et al. 1983).

The single-stranded RNA genome sequence of the type isolate of CPMMV is ~8,000 nucleotides (nt) in size, not taking into account the 3' terminal polyA tail (Menzel et al., 2010). The capsid protein monomer is ~32-36 kDa in size. The genome encodes six ORFs with an arrangement like members of the genus, *Carlavirus* (family, *Betaflexiviridae*; order *Tymovirales*). The CPMMV genome has been shown to undergo recombination, with the events detected thus far, occurring primarily in the polymerase gene and less frequently in other regions of the genome (Zanardo et al., 2014a).

**Historical overview.** *Cowpea mild mottle virus* (CPMMV) was first identified in Ghana during 1973 when it was detected in symptomatic cowpea (*Vigna unguiculata* L.) plants. The virus was found to be seed-borne to varying extents in three leguminous hosts examined, ranging from nearly 100% in soybean (*Glycine max* Merr.) and cowpea, to 1-4% incidence in common bean (*Phaseolus vulgaris* L.) (Bock et al. 1975). Plants developing from CPMMV-infected seed were primarily asymptomatic or symptoms were inconspicuous, therefore, the virus was considered to be of only minor importance (Brunt and Kenten, 1973). Later, it was recognized that the disease of groundnut referred to as 'Ngomeni mottle' (Storey and Ryland, 1957) was caused by CPMMV, thus apparently the virus has been present in legume crops in Africa for a longer period of time than was initially realized. Also, outbreaks of CPMMV were reported in peanut (groundnut) (*Arachis hypogaea* L.) in Kenya and Tanzania during 1974-77 (Bock et al, 1975, 1977), and thereafter the disease was recognized as an impending, persistent threat to peanut production there (Bock and Waiigai, 1984). Soon after, CPMMV was reported in India and throughout Southeast Asia where it infected peanut and soybean crops (Iizuka et al., 1984; Iwaki et al., 1986). During 1992-94 the first outbreak of CPMMV disease occurred in Sudan, causing heavy losses (10-100%) in the irrigated peanut crop, grown along the

Blue River and the White Nile River, however, rain-fed crops were found to be unaffected. Early-stage infected peanut plants produced no pods whereas, those infected during or after flowering experienced losses as great as 60%. The outbreak in Sudan was associated with heavy whitefly vector infestations and represented the first report of the virus having reached major economic importance in peanut (El-Hassan et al., 1997). Although CPMMV now occurs in at least 27 countries and on all continents where legumes are grown, the distribution and damage caused by the virus has not been determined in a comprehensive manner, suggesting that it probably occurs more widespread and in previously unreported locations than is currently known.

***Current status in soybean in the Americas.*** Soybean (*Glycine max* Merr.) is the most important grain legume crops grown throughout the world for human consumption, being used for animal feed, oil production, confectionaries, and human consumption, among other purposes. Weeds, insect pests, and diseases are the major biotic stresses that limit soybean production. In recent years attempts have been made to develop stress tolerant/resistant and high yielding soybean varieties by several biotechnology companies. In addition to infecting legume crops in Africa and elsewhere worldwide where CPMMV occurs naturally, or has been introduced, most recently, CPMMV has become the primary yield-limiting virus infecting the soybean crop in Argentina and Brazil, since it was first identified in soybean in 2000-01 in Goias State (Almeida et al. 2003; 2005) causing stem necrosis in widely-grown genotypes (Zanardo and Carvalho, 2017). It should be noted that the first detection of CPMMV in Brazil was made in common bean (Costa et al., 1983), suggesting that at least some variants of CPMMV could have been introduced there on infected bean seed long ago. Currently, it is not possible to rule out the possibility that the introduction of the stem necrosis variant occurred recently by way of infected soybean seed, however, disease emergence may be related to plant genotype and/or to viral diversification that has led to the occurrence of a new virus variant, or both. Indeed, a number of previously unknown host plant species have been identified, including chia (*Salvia hispanica*, Lamiaceae) (Celli et al. 2016) and dixie ticktrefoil (*Desmodium tortuosum*, Fabaceae) (Henning et al. 2014).

Little to no trace-back has been reported that could help identify the prospective source(s) of the infected seed, but if accomplished, the information could provide valuable epidemiological verification with great relevance to soybean and other legume breeding programs, as well as to diagnostic development.

In Puerto Rico, soybean is cultivated in winter nurseries to advance breeding programs by the United States Department of Agriculture, U.S. universities, and several seed companies. However, this effort has become constrained by widespread CPMMV infection of soybean plantings, placing a major limitation on the use of this valuable location for breeding objectives and for increasing seed during the winter months. During 2010 symptoms of leaf vein and stem necrosis, and plant stunting were observed in soybean experimental plots in the municipalities of Juana Diaz, Santa Isabel, Isabella, and Guayanilla. In one winter nursery in Santa Isabel, 100 percent incidence was observed in certain of the soybean lines, indicating the extreme potential of this disease to undermine the present and future cultivation of soybean in Puerto Rico either in winter nurseries or during other times of the year (Rodrigues et al., 2014).

During 2010, 100% infection by CPMMV was documented in a number of soybean lines planted at Juana Diaz, Puerto Rico by the Illinois Crop Improvement Association. At the same time, virus infection were observed in experimental soybean field blocks located at the Agricultural Experimental Station in Juana Diaz, Puerto Rico. Preliminary observations indicated that owing to the magnitude of the losses, the outbreak was likely associated with infections that occurred during early stages of growth, and most probably, was seed borne.

## II. CPMMV Disease Symptoms

The symptoms associated with CPMMV of soybean plants from Puerto Rico include: foliar chlorosis, foliar necrosis, and stunting of plants are shown in [Figures 3-5](#). Infection by CPMMV causes similar symptoms but with varying severity and extent of yield loss in bambara groundnut, groundnut (peanut), soybean, and winged bean in Cote d'Ivoire, Ghana, India, and Indonesia (Fauquet et al., 1979; Thouvenel et al., 1982; Fauquet and Thouvenel, 1987; Offei and Albrechtsen, 2005). The basis for the wide range of responses by plant hosts to CPMMV infection has not been determined. The differential symptom development observed in the same host species is thought to be due to differences in virulence between the viral isolates themselves, to the differential susceptibility among the different host plant species and their respective cultivars, and/or to widely variable environmental conditions that prevail in the different CPMMV affected locales (Rosario et al., 2014; Jeyanandarajah and Brunt, 1993; Zanardo et al., 2014b). For example, CpMMV isolates from Israel and Ghana infect members of the Solanaceae (Antignus and Cohen, 1987; Brunt and Kenten, 1973), while the Florida and Brazilian, Florida, Iran, and Thailand isolates of CpMMV do not (Almeida et al., 2005; Iwaki et al., 1982; Tavasoli et al., 2008). Despite these differences, CPMMV isolates thus far have not been distinguished by electron microscopy or serologically, suggesting that host range and possibly RNA sequence divergence will provide the most robust differentiation of isolates, with extent of genome divergence potentially serving as one of the most important means of virus identification, together with biological characteristics.

In Puerto Rico the soybean isolates of CPMMV cause dwarfing, shoot die back, and stem necrosis. The major outbreaks there have been in soybean nurseries grown for seed production, making the extensive damage intolerable. Thus far, symptoms of leaf vein and stem necrosis and plant stunting were observed in soybean experimental plots in the municipalities of Juana Diaz, Santa Isabel, Isabella and Guayanilla, Puerto Rico. Observations in a 2005 winter nursery in Santa Isabel showed 100 percent incidence in some soybean lines, indicating the potential of damage of this disease in soybeans. Symptoms were associated with flower dropping and poor development of the pods. Seed harvested from infected plants showed severe deformation and reduced weight, compared to seeds from healthy, virus-free plants (Rodrigues et al., 2008).

## III. Biology, Spread, and Risk

**Host range.** Natural hosts include *Canavalia ensiformis*, groundnuts (*Arachis hypogaea*), *Phaseolus lunatus*, *P. vulgaris*, *Psophocarpus tetragonolobus*, soybeans (*Glycine max*), tomatoes (*Lycopersicon esculentum*), *Vigna mungo*, probably eggplant *Solanum melongena*, the cowpea species: cv. Blackeye (*Vigna unguiculata*), *Vicia faba* and *Vigna subterranean*, and bean, *Vigna unguiculata* subsp. *Sesquipedalis*. The virus also occurs in various weeds

(Fabaceae), including *Stylosanthes* and *Tephrosia* spp. Many more hosts can be artificially inoculated (Brito et al., 2012, Rodrigues et. al., 2014).

**Vector transmission.** The whitefly *B. tabaci* (Genn.) is reported to be the only insect vector of CPMMV. The whitefly vector transmits the virus in a non-persistent manner (Muniyappa and Reddy, 1983), and therefore is non-circulative and unlikely to be transovarially transmitted. The first report of experimental whitefly transmission was from Japan with an isolate present there (Iwaki et al., 1982). Also, *B. tabaci* was shown to transmit isolates of CPMMV from Israel (Cohen and Antignus, 1982), Brazil (Costa et al., 1983), India (Muniyappa and Reddy, 1983; Yadav et al., 2013), Nigeria (Rossel and Thottappilly, 1993; Anno-Nyako, 1986; Thottappilly and Rossel, 1992), Indonesia (Saleh et al., 1989), and Jordan (Monsour et al., 1998). These reports corroborate the association of whitefly with the disease outbreak in Sudan peanut crops during 1992-94 (El-Hassan et al., 1997). In Brazil the B biotype of *B. tabaci* was identified as the vector of CPMMV there (Marubayashi et al., 2010). CPMMV was successfully transmitted by grafting, mechanical inoculation, and by the B biotype (J.K. Brown, unpublished results) of the whitefly *B. tabaci* in Puerto Rico (Rodrigues et al. 2008, Rodrigues et al. 2014)

**Seed transmission.** The Ghanaian isolate, obtained from a seed-infected cowpea seedling in a vegetable garden, was subsequently shown to be seed-transmitted to unspecified levels in cowpea, soybean and French bean (Brunt and Kenten, 1973). CPMMV is also seed-transmitted in other plant species and other countries. Several preintroductions of cowpea lines tested from Botswana, India, and Kenya were shown to harbor CPMMV (Gillaspie et al., 1995). However, reported levels of seed transmissibility of CPMMV isolates in different host species and countries are contradictory. Thus the virus was reported to be seed-borne to a level of 1-3% in cowpea in India (Nain et al., 1994), to 0.9% in soybean in Thailand (Iwaki et al., 1982), to 0.05-1.66% in 25 soybean cultivars in India, to 8 of 27 soybean cultivars in India (Yadav et al., 2013), to 6-21% in bambara groundnut in the Cote d'Ivoire (Fauquet and Thouvenel, 1987) and to unreported levels in soybeans in the Cote d'Ivoire (Fauquet and Thouvenel, 1987) and in cowpea in India (Mali et al., 1987), Burkina Faso and Swaziland (Hampton et al., 1992). A severe strain of CPMMV was shown to be seed-borne in groundnut in India (Sivaprasad et al., 1990). Thus far, no seed transmission has been demonstrated for the Puerto Rican CPMMV isolate, however, soybean seeds from virus-infected plants were shriveled and smaller compared to seeds from virus symptom free plants (Fig. 1). The symptoms that developed following graft-inoculation (Fig 2B), whitefly-mediated transmission (Fig. 2 A), and mechanical inoculation (Fig. 2C) were like those observed in the field-infected plants.

**Epidemiology.** The epidemiology of CPMMV has yet to be investigated in a comprehensive manner. The relative importance of infection in weeds and seed transmission as primary sources of inoculum for crop infection especially needs to be studied further, as does the importance of seed transmission in the accidental dissemination of virus from currently infected locales, some of which are endemic and others resulting from exotic introductions. Perennial weed species naturally infected by CPMMV are possibly important sources of infection for both tomatoes and leguminous crops in Jordan, Kenya, Nigeria and India (Anno-Nyako, 1984; Bock and Waiigai, 1984; Bock et al., 1975, 1977, 1979; Muniyappa and Reddy, 1983; Monsour et al., 1998). Similarly, seed transmission is known to occur in a large number of diverse legume crops. Therefore, it seems likely that plants that become infected through the seed would seem likely to serve as primary sites of infection for further spread by whiteflies within and to adjacent susceptible crops and weeds. The seed borne nature of the virus in cowpea and common bean,

however not confirmed in soybean in Puerto Rico, suggests that the potential is great for inter-regional and international dissemination of CPMMV.

#### **IV. Identification of CPMMV Strains, Detection, and Monitoring**

**Taxonomy and identification.** CPMMV is a species in the genus, *Carlavirus* (family, *Betaflexiviridae*; order *Tymovirales* (Adams et al., 2005). Viruses (or diseases) such as *Groundnut crinkle virus* (Dubern and Dollet, 1981), *Psophocarpus necrotic mosaic* (Fortuner et al., 1979) and *Voandzeia mosaic virus* (Fauquet and Thouvenel, 1987) in Cote d'Ivoire, Tomato pale chlorosis disease in Israel (Cohen & Antignus, 1982), Tomato fuzzy vein disease in Nigeria (Brunt & Phillips, 1981) and *Bean angular mosaic virus* in Brazil (Costa et al., 1983; Gaspar et al., 1993) were shown to be serologically most closely related to CPMMV, and are so have been grouped under the same species name of CPMMV. Virus isolates from solanaceous host species in Jordan and Israel, although seemingly similar serologically and with respect to biological characteristics to West African and Indian legume isolates, have been considered as distinct strains, however, additional information is needed to clarify the identity and taxonomy of this group of isolates/strains (Menzel et al., 2010). Differences in the virulence of CPMMV isolates occurring elsewhere in additional countries also have been noted (Anno-Nyako, 1986, 1986, 1987; Siviprasad and Sreenivasulu, 1996).

Although CPMMV is classified in the *genus*, *Carlavirus* (Adams et al., 2005; King et al., 2011; Naidu et al., 1998), it has been shown to be serologically unrelated to 20 or more aphid-transmitted carlaviruses (Brunt and Kenten, 1973; Adams and Barbara, 1982; Brunt et al., 1982; Gaspar and Costa, 1993). This may not be surprising because CPMMV also differs from all other known carlaviruses that are transmitted by the whitefly *Bemisia tabaci* (Genn.) sibling species group (Brown, 2010). Further it produces brush-like inclusions (Brunt et al., 1983; Thongmeeakom et al. 1984; Gaspar and Costa, 1993) that are unique among carlaviruses described thus far. And unlike the majority of aphid-transmitted carlaviruses, CMMV is seed-transmitted (El-Hammady et al., 2004). Thus, there remains some controversy as to whether CPMMV is a carlavirus, or a member of a new genus that is closely related to carlaviruses (King et al., 2011).

Also, distinguishing species, strains, of variants has been confounded by evidence for variation between isolates otherwise identified as CPMMV. For example, CPMMV isolates originating from solanaceous hosts in Jordan and Israel appeared to be very similar to each other, but were distinct from selected legume isolates from India and West Africa. An immune-electron microscopy decoration study indicated that a legume isolate of CPMMV from Brazil was serologically, distantly related to two other legume isolates but strikingly dissimilar to a Jordanian isolate. Moreover, CPMMV isolates from Jordan and Israel differed from the legume isolates by inducing only banded or non-banded virion aggregates but no brush-like virion aggregates, as was reported to be characteristic of legume isolates of CPMMV from Africa, Thailand and Brazil. Under the conditions of the present study brush-like inclusions were also found with Indian and West African isolates (Monsour et al., 1998).

Analysis the predicted amino acid sequences of carlaviruses (Menzel et al., 2010) indicated that CPMMV shares 46-59% identity with 8 aphid-borne carlaviruses, thereby falling below the 80% cutoff for



species demarcation (Adams et al., 2005), adding support for the recognition of CPMMV as a distinct carlavirus species.

The 3'-untranslated region of 120 nt shares 78-92% identity (Badge et al., 1996; Gaspar et al., 2008; Naidu et al., 1998) with seven isolates for which partial CPMMV sequences are available in Genbank (ICTV website; NCBI GenBank database). By comparison, a recently discovered CPMMV isolate infecting soybean in India shared only 75-79% nt identity with the other seven known isolates (Yadav et al., 2013). In the US, Rosario et al. (2014) reported a CPMMV isolate from bean and wild plants, and from whitefly samples collected in Florida. Based on the coat protein gene, the Florida-US isolate was most closely related to isolates from Ghana, India, South America (Brazil), and the Caribbean (Puerto Rico) region. Similarly, Zanardo et al. (2014b) reported that the replicase gene of the CPMMV isolate occurring in soybean in Brazil shared only 61% pairwise amino acid sequence homology with the isolate from Ghana, indicating important differences between these viral genomes.

An assessment of genomic variability for 18 CPMMV isolates from soybean grown in different Brazilian states (Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais, Pará) during 2001 and 2010, exhibiting a variety of symptoms. Symptoms in one soybean variety cv. CD206 ranged from 'mild' [crinkle/blistering leaves, mosaic and vein clearing] to 'severe' [bud blight, dwarfing, leaf and stem necrosis] (Zanardo et al., 2014). Recombination analysis predicted that one CPMMV isolate was recombinant. Pairwise comparisons and phylogenetic analysis of partial genomes (ORF 2 to the 3' terminus) and for ORFs 2 to 6 separately, suggested the virus comprises distinct isolates, referred to as: CPMMV-BR1 and CPMMV-BR2. The isolates were grouped phylogenetically based on symptom phenotype in soybean, and not by year or geographical location of the sample from which each sequence was determined (Zanardo et al., 2014b). A similar conclusion with respect to divergence and evidence for two distinct CPMMV strains can be drawn from the phylogenetic tree reconstructed from sequences available in Genbank (though 2016), analyzed independently by the authors of this study (Figure 6).

**Detection and monitoring.** The ability to formally detect and monitor CPMMV in the US mainland and elsewhere in Puerto Rico has been minimal owing to its recent introduction into the Americas. An isolate of CPMMV identified in Florida was detected using a genomic pathology approach with results validated by RT-PCR amplification (Rosario et al., 2014). In Puerto Rico, bean, soybean and other legumes grown there in winter nurseries have recently been found to be infected by the virus. And, a molecular diagnostic assay has been implemented, however, the ability to differentiate strains and optimize detection in different host species have not been addressed for these isolates (J.C. Rodrigues, unpublished results).

CPMMV detection has primarily been accomplished using serological and most recently, molecular methods. Monsour et al. (1998) demonstrated biological and serological differences between CPMMV isolates from leguminous and solanaceous hosts. A polyclonal antibody raised to the coat protein of a Brazilian isolate of CPMMV (overexpressed as a fusion protein in *E. coli*) was demonstrated to detect the virus using an enzyme-linked immunosorbent assay (ELISA) and by Western blot (Carvalho et al. 2013). Tavassoli et al (2009) developed ELISA and reverse-transcriptase polymerase chain reaction (RT-PCR) approaches to detect CPMMV isolates in soybean. Gaspar et al., (2008) has developed a degenerate primer that facilitated amplification of

a portion of the 3' terminus of three distinct carlaviruses. Most recently, RT-PCR amplification of a fragment of the coat protein gene was developed by a commercial source (AGDIA, Inc., Elkhart, IN, USA) with primers based on Maroon and Zavriev (2002).

**V. Response** (portions of this section are modeled or excerpted after the Red Leaf Blotch of Soybean Recovery Plan; see Hartman 2011).

Currently, there has been no formal response to the potential for or ramification of the outbreak that has become persistent in legume species grown in Puerto Rico, Mexico, and the US mainland (Florida).

In general, once the detection of a high risk and/or select agent pathogen is confirmed by a USDA, APHIS, PPQ recognized laboratory, APHIS, in cooperation with the State Department of Agriculture, is responsible for the 'response'. The response involves deployment of teams of experts and survey personnel to the site of the initial detection to conduct investigations and initiate delimiting surveys. Actions may include: (1) regulatory measures to quarantine infested or potentially infested production areas to prevent infected material from being transported outside the zone, and (2) control measures which may include host removal and destruction, and/or ensuring adherence to required sanitary practices. APHIS imposes quarantines and regulatory requirements to reduce or negate importation and interstate movement of quarantine-significant diseases or regulated articles, and works in conjunction with the respective state(s) to impose them together with state regulatory actions that can restrict intrastate movement.

After the results of a delimiting survey are known, if the disease (viral pathogen) is limited in distributed in commercial and non-commercial plant hosts, options for eradication include destruction of the crop and protective insecticide applications to kill the whitefly vector in the vicinity. If the infection is widely distributed, it is likely that the entire crop in all infected locations would be destroyed. Such measures, or others, will be determined by USDA-APHIS.

**VI. USDA Pathogen Permits and Regulations** (portions of this section are modeled or excerpted after the Red Leaf Blotch of Soybean Recovery Plan; Hartman 2011).

Until recently, no specific regulations have been established regarding detection, response, or recovery from the introduction and spread of CPMMV in the U.S. However, as a result of its newly recognized risk potential, CPMMV has been placed on the Regulated Plant Pest list maintained by the US Department of Agriculture, Animal and Plant Health Inspection Service.

Permit and registration requirements for plant diseases and laboratories are regulated under two authorities: The Plant Protection Act of 2000 (codified at 7 CFR Part 330) and the Agricultural Bioterrorism Protection Act of 2002 (codified at 7 CFR Part 331). Laboratories receiving suspect CPMMV-infected plant materials are required to have PPQ permits. Laboratories possessing, using, or transferring high risk and select agents must be registered; however, diagnostic laboratories that identify select agents or toxins are exempt from this requirement as long as they complete an APHIS/CDC Form 4 and destroy the culture within 7 days.

The permit requirements of the Plant Protection Act apply to all pests of plants or plant products. This includes importation and interstate movement of pure cultures, arthropod vectors of plant pathogens, diagnostic samples, and infected plant material. The movement of infected plant material, regardless of the pest's quarantine status, requires that the receiving laboratory to have a permit. The importation and/or interstate movement of soil is similarly regulated when the intent is to isolate microbes that may be pests of plants or plant products. For guidance on the permitting of plant pests and soil samples, consult the PPQ permit website available at: <http://www.aphis.usda.gov/ppq/permits/> or contact PPQ Permit Services Customer Services at (301) 734-0841.

The Agricultural Bioterrorism Protection Act specifies requirements for possession, use, and transfer of organisms listed as select agents or toxins, such as the newly listed CPMMV. Once an unregistered diagnostic laboratory identifies a presumptive select agent or toxin, they must immediately notify the Agriculture Select Agent Program (ASAP), complete an APHIS/CDC Form 4 within 7 days, and either destroy or transfer the agent to a registered entity within 7 days (prior approval of the ASAP required). If a diagnostic laboratory held part of a screened sample (or culture) for voucher purposes, and the sample forwarded to the USDA Beltsville Laboratory was identified as positive for the select agent, then the USDA Beltsville Laboratory will notify immediately both the ASAP and the sending diagnostic laboratory that a select agent has been identified. The USDA Beltsville Laboratory will submit the APHIS/CDC Form 4 within seven days, and all unregistered labs will either destroy, or transfer the samples to a registered entity, within 7 days of the receipt of the results. The Agriculture Select Agent Program personnel must witness the destruction of the sample(s) or culture(s) within that time period. Clarification of these requirements and other information related to adherence to the select agent regulations is available at: [http://www.aphis.usda.gov/programs/ag\\_selectagent/](http://www.aphis.usda.gov/programs/ag_selectagent/) and <http://www.selectagents.gov>, or call (301) 734-5960, Agriculture Select Agent Program.

## **VII. Economic Impact and Compensation**

Yield losses due to CPMMV infection of legume crops, in general, vary widely, and can range from 10-80% depending on virus isolate, host species/genotype. Information about the potential impact of the virus has been reported primarily from Africa and India, where experience with the virus has been longstanding. In contrast, yield loss in soybean crops in Argentina and Brazil have been extreme in some genotypes, particularly those expressing stem necrosis, and losses ranged from 10-100%, following presumed introductions of CPMMV on seed. Recent statistics from affected areas in sub-Saharan Africa where legumes are widely planted mostly in smaller acreages are partially available, and the disease has continued to persist (Taiwo, 2003; Time et al., 2010). Recent estimates of crop damage by putatively introduced isolates have been best documented based on recent outbreaks in South American countries, where a major breeding program was initiated, particularly in Brazil, to combat the virus disease (Arias et al., 2015).

Among the potential hosts of CPMMV in the U.S. soybean is the crop at greatest risk, followed by common bean (dry and fresh marker), cowpea, garbanzo bean, and peanut, particularly when the respective crop is grown in mild climate locales where the whitefly vector is endemic and typically abundant during the warmest part of the growing season. Soybean in the US (2012) is

planted to approximately 75,000,000 acres valued at about 37 billion dollars). (<http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/related-data-statistics.aspx>), and in some instances, is grown where the climate is mild. Common bean (fresh and dry), cowpea, and garbanzo beans (chickpeas) account for variable, but substantial acreages of legume production in the US (<http://www.nass.usda.gov/>), and also are at risk, particularly where the whitefly vector is endemic. However, all areas, regardless of the climate, are susceptible to the introduction of CPMMV through seed.

Because US germplasm varies widely, and likely is quite different in some parts of the country compared to South American genotypes, it is difficult to estimate the potential damage. The use of forecasting, based on the most likely susceptible areas for establishment, would aid in arriving at a more refined estimate of impacts. This includes the potential range of the whitefly vector, which in recent years, has extended northward in the US. Also, whether insecticides applied to soybean crops to control other pests and vectors, would be expected to be effective against whitefly if it invaded soybean-growing areas. Because nothing is known about the epidemiology of the mild mottle disease in legumes grown in US conditions (given the disease has not emerged), extrapolations taken from other affected locales could provide useful measures of risk and likelihood of spread, and whether it could become a persistent viral pathogen. And if so, in what areas of the US over others might it persist from season to season, compared to those from which it readily eradicated if introduced on seed.

Currently there is no test established to detect this virus in commercial seed or in germplasm/seed increases carried out in the US and in offshore winter nurseries where the virus is already known to occur. Compensation by the Risk Management Agency (RMA) for a loss caused by CPMMV would likely become available, if the producer can confirm that available control measures were taken. This would imply that certified clean seed is made available. If the insured does not provide evidence of procurement of clean seed, then it would seem unlikely that insurance coverage would be provided.

## **VIII. Mitigation and Disease Management**

The vulnerability of the legume crop to infection by this whitefly-transmitted virus is of particular concern because it is both seed and whitefly vector borne, indicating that even a very low level of seed transmission followed by whitefly transmission could lead to rapid and widespread re-distribution, and advanced spread of CPMMV in a relatively short time, if not detected early, and then left unmanaged. Any mitigation strategy that is considered and implemented must be coordinated among Federal, State and local regulatory officials because efforts by private individuals or industry are not expected to provide widespread disease control.

***Prevention and exclusion.*** CPMMV is not yet reported in soybean or any other legume or non-legume host in the mainland USA, but the risk for its introduction in legume growing areas seems quite high because seed that is increased offshore, or that is the product of breeding programs from infected locations (in Africa, Argentina, Brazil, perhaps Mexico, and Puerto Rico) likely makes it way to the US for spring-summer season breeding activities. Thus, phytosanitary regulations are needed. If CPMMV is discovered to already occur in the USA, it probably has high potential for rapid spread and could be difficult to control, at least at the

outset, particularly in areas where the whitefly vector is abundant. Therefore, exclusion of this disease through port activities is an essential initial step in the mitigation and disease management strategy.

**Diagnostics development.** The lack of understanding of viral genomic diversity has hindered the development of diagnostics tests to ensure virus detection when it is present. Therefore, research efforts are needed to better understand the ecology, epidemiology, genetics, and population biology, and risks posed by CPMMV on a global basis. Without such knowledge, developing and sustaining durable virus-resistant varieties would seem to be a difficult undertaking.

**Biological, chemical, and cultural control.** Integrated approaches should be used to management of this aggressive viral pathogen, however, it should be noted that mitigation and management approaches that specifically address CPMMV have not been previously addressed for US crops. Based on knowledge of other seed-borne viruses, plant viruses transmitted in a non-persistent manner by the whitefly vector, *B. tabaci*, and efforts in Brazil to develop resistance in soybean, management practices can be extrapolated to apply to CPMMV outbreaks in the US, depending on the plant host species that are involved. As such, short-term approaches would include control of the whitefly vector using appropriate insecticides (Belay et al. 2012) together with biological control using natural enemies when feasible. Medium-term approaches would rely on establishment and enforcement of clean seed testing programs for common bean, cowpea, garbanzo, and soybean seed to be planted in the US. In addition, once diagnostic tests are available to detect all introduced strains (and differentiate between other exotic strains, as is possible), removal or management of weeds and other alternate hosts (solanaceous hosts, ornamentals, and other minor crop or legume host species) identified as such, appropriate management strategies would be practiced within the outbreak zone. In the long term, once the virus has been introduced, virus-resistant varieties will be essential to protect major legume crops from becoming infected and/or serving as a virus source for other crops in the vicinity. An alternative plan may be needed for winter nursery screening and seed increases currently carried out in Puerto Rico and Mexico, in that seed will need to be rid of virus, and a routine diagnostic testing implementing, together with strict quarantine to prevent new introductions. It may be necessary to shift soybean and other seed programs to areas where WFs and those weeds are less problematic, however, it is difficult to envision where such a location exists that also would not eventually become contaminated due to exotic introductions if clean seed enforcement is not practiced in general. Otherwise, a comprehensive revision of seed phytosanitary protocols should be established to reduce the incidence of the virus in fields grown for seed multiplication.

**Germplasm and CPMMV disease resistance.** Sources of resistance to CPMMV in legumes are scarce. Brace (2012) reported that because the high incidence of CPMMV-like symptoms occurring in Puerto Rico and Mexico, allowing reliable phenotypic data to be collected and some inferences about the inheritance to CPMMV-like in soybean to be reached. Phenotypic segregation of F<sub>2</sub>-derived lines fit a 3 susceptible to 1 resistant segregation ratio, and all F<sub>1</sub> plants evaluated became infected. Based on the segregation ratio and phenotype of F<sub>1</sub> plants, CPMMV-L resistance was controlled by a single recessive allele designated as *rbc1*. Molecular mapping further confirmed a single gene model by identifying only one significant region on Chromosome 18 (LG G). Location of the locus was narrowed to a 2.4 cM region flanked by the SSR markers, BARCSOYSSR\_18\_0456 and BARCSOYSSR\_18\_0458. Suryanto et al. (2014)

suggested that soybean resistance for an Indonesian CPMMV isolated was additive and controlled by two duplicate, recessive epistasis genes.

While the main soybean and most other legume-growing states in the US are temperate, CPMMV could become a threat to fresh or dry bean production in at least some of those locations, even if only as a seasonal pathogen, when introduced through seed. If isolates not restricted to legumes are involved, vegetable and ornamental crops could also be affected. Although the epidemiology of CPMMV in temperate climates has not been studied, it seems likely that the virus could survive from season to season in the mild climate zones in the US, after an accidental introduction by seed or even the whitefly vector harboring the virus ingested from various species of infected plants (asymptomatic or symptomatic). Indeed, an isolate of CPMMV has already been identified in whiteflies from Florida (Rosario et al. 2014), however, the exact origin of this isolate has not been determined, nor is it known if CPMMV is more widely distributed in Florida or other Sunbelt states where whiteflies thrive, than thought.

**Education.** Education efforts to raise awareness of CPMMV symptoms, host range, and transmission modes i.e. by seed and the whitefly vector, *B. tabaci*, are needed. Importers, plant breeding programs, growers, diagnosticians, and cooperative extension experts must be made aware of the disease and trained to identify the virus, once diagnostics are available. Information on how to distinguish this virus from others that cause similar symptoms, particularly in legumes, will be critical for detection at ports of entry and in the field. These measures will be instrumental in providing additional information about the threat posed by CPMMV, and downstream management practices that are put into place to mitigate further introductions and/or spread from infected fields.

## **IX. Current Infrastructure, Needs and Experts**

**Infrastructure.** On the mainland USA there is no current effort devoted to research or breeding for resistance to CPMMV. However, upon identification of CPMMV in local fields, research has been underway at the Agricultural Experimental Station (AES) System, University of Puerto Rico. The virus has been identified in two areas previously dedicated to growing *Phaseolous* species and winter soybean seed multiplication nurseries located in both the southern and northern parts of the island. These AES were the first sites where the disease was observed in Puerto Rico. Collaborative studies were carried out by UPR-AES researchers and seed companies having mutual concerns about the potential threat and/or immediate impact of the disease on the soybean crop in USA and elsewhere, where the seed will be grown for research purposes (breeding, variety trials) and/or for commercial production. One option for furthering the research findings could involve a collaborative effort between the University of Puerto Rico and APHIS, utilizing the newly constructed, certified quarantine facility laboratory and greenhouse facilities (10,000 sq. ft.) at the Agricultural Experiment Station, Rio Piedras, Puerto Rico.

**CPMMV Experts:** Because of the impact of the disease associated to soybean described in South America, efforts to characterization of the virus, vector relationship and breeding for resistance have been so far mostly conducted at research agencies and agricultural universities in Brazil.

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## **X. Research, Extension, and Educational Priorities for CPMMV in Legumes and Other Susceptible Plant Species**

- Immediate efforts should be undertaken to determine if CPMMV is seed borne (presence in seed lots produced in offshore infected areas) in imported soybean seed, common bean, cowpea, and other susceptible legumes. If so, what is the anticipated risk?
- Education (outreach, pamphlets with symptoms on various hosts, distribution) to alert producers, educators, diagnosticians, scientists, and regulators to recognize the symptoms of CPMMV disease in its numerous hosts (not only legumes), and its likely risks, including introduction and transmission through seed, and of spread by the whitefly vector in mild climate locales of the US mainland.
- Training materials are advised for port of entry Safeguarding Specialists (USDA) and Department of Homeland Security personnel, and First Detectors.
- Determine the identity and distribution of CPMMV isolates in the US particularly in mild climate areas of the country where the whitefly vector is endemic, and where legume crops are grown from seed produced in CPMMV-infected areas.
- A better understanding of viral genomic diversity is advised to guide the development of diagnostics tools to ensure that all variants are detectable.
- Assessment of the ecology, epidemiology, potential sources of host-plant genetics for improvement, and overwintering capacity of CPMMV have not been assessed in the US. The most vulnerable crops are thought to be soybean, and then common bean, cowpea, as well as non-legume host (particularly, Solanaceae) species grown in the mainland US.
- Screening for sources of resistance in soybean and other legume species of importance to the US, including common bean and peanut, is advised for preparedness against impending outbreaks.

- A certified, clean seed program is advised to reduce risk imposed by seed transmission, which even if at a low percentage overall, could lead to rapid spread of CPMMV by the whitefly vector.
- Sampling protocols, especially for seeds, are needed to ensure accurate diagnostic with acceptable level of detection and confidence. This may also include protocols for testing the whitefly vector.
- Whitefly management will require use of insecticides, biological control, and/or biopesticides, and management of weeds and other alternate hosts. Whether registration of compounds would be required to manage whitefly in soybean or other legumes needs to be determined.

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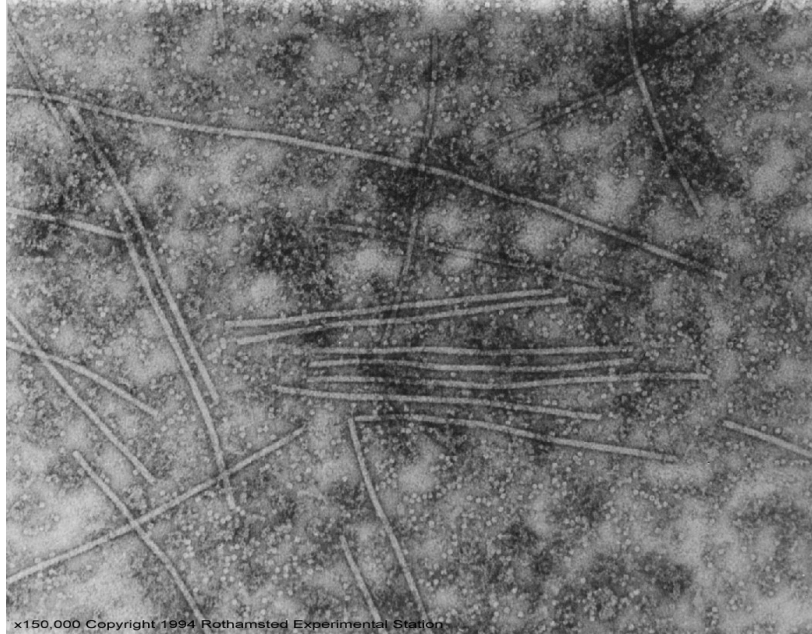
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**Table 1.** Host plants collected near soybean fields and screened with ELISA for *Carlavirus*. Santa Isabel, Puerto Rico (Rodrigues et al. 2014).

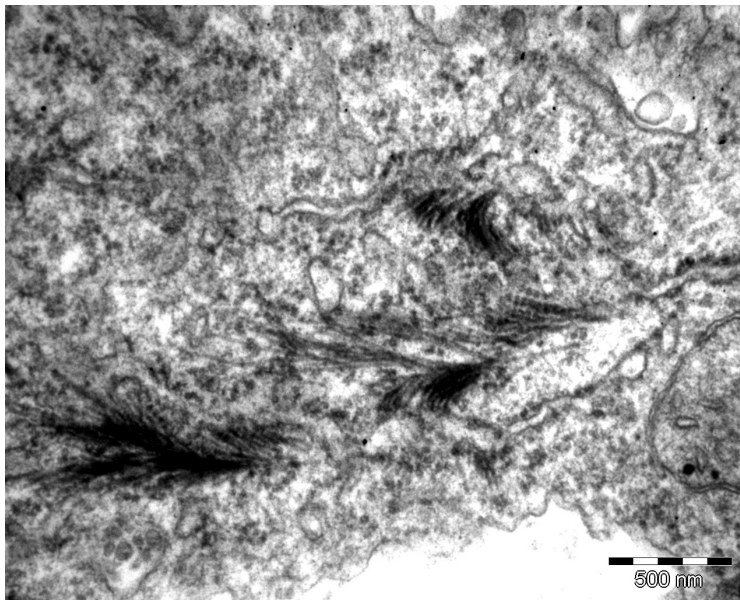
Scientific name	Family	ELISA result	# plants tested	% Incidence
<i>Ludwigia octovalvis</i> (Jacq.)	Onagraceae	+	4	25
<i>Solanum americanum</i> Mill.	Solonaceae	+	4	25
<i>Triantheme portulacastrom</i> L.	Aizoaceae	+	4	25
<i>Ipomea</i> sp.	Convolvulaceae	+	4	25
<i>Boerhavia erecta</i> L.	Nyctaginaceae	+	4	25
<i>Argemone Mexicana</i> L.	Papaveraceae	+	4	50
<i>Euphorbia heterophylla</i> L.	Euphorbiaceae	–	4	0
<i>Amaranthus dubius</i> Mart.	Amaranthaceae	–	4	0
<i>Malvaceae</i> sp.	Malvaceae	–	4	0
<i>Datura stramonium</i> L.	Solonaceae	–	4	0
<i>Cleome gynandra</i> L.	Cleomaceae	–	4	0
<i>Kallstromia maxima</i> L.	Zygophyllaceae	–	4	0
<i>Macroptilium lathyroides</i> (L.) Urban	Fabaceae	–		0
<i>Vigna unguiculata</i> L.	Fabaceae	+	8	25
<i>Lycopersicon esculentum</i> L.	Solonaceae	–	4	0
<i>Cucurbita</i> sp.	Cucurbitaceae	–	4	0
<i>Ipomoea batatas</i> (L.) Lam.	Convolvulaceae	–	4	0
<i>Glycine max</i> (L.) Merr.	Fabaceae	+	8	25

## Figures

**Figure 1.** Transmission electron micrograph of *Cowpea mild mottle virus* slightly flexuous rods ~ 650-700 nm x 13 nm diameter (Courtesy, Phil Jones, Rothamsted Experiment Station, UK).

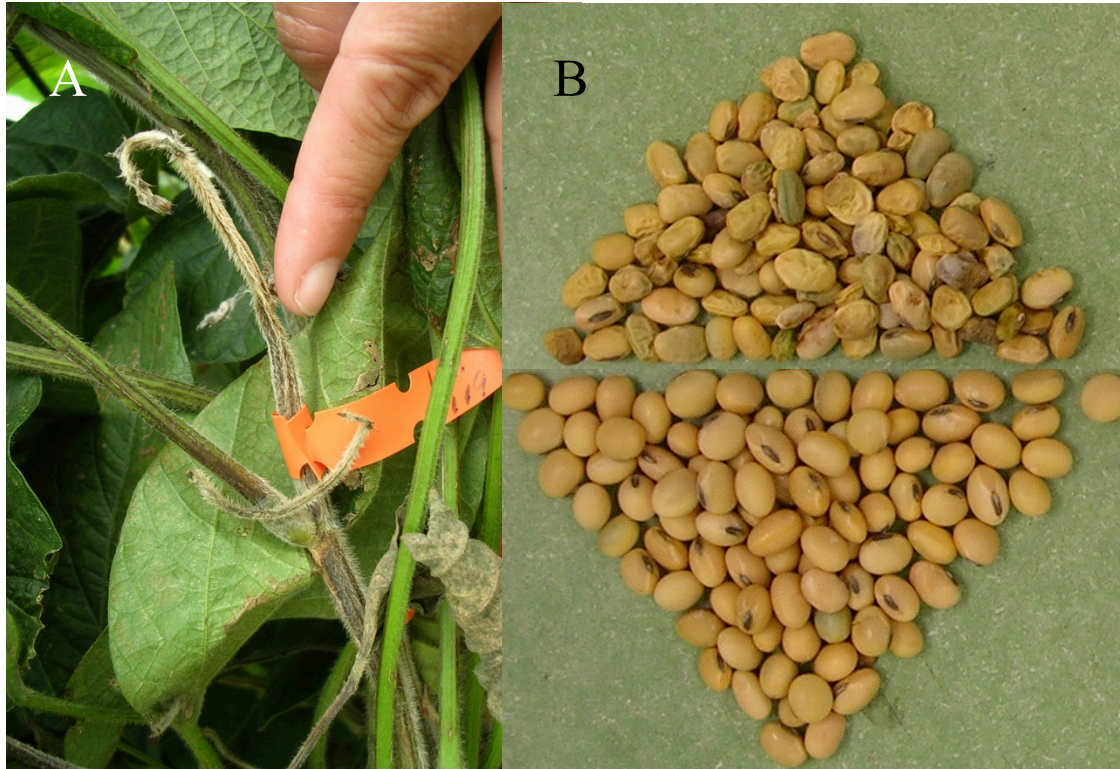


**Figure 2.** Feather- or brush-like inclusions in the cytoplasm of CPMMV-infected leaf parenchyma cells. A similar type of inclusion body has been observed for other members of the genus, *Carlavirus*, transmitted by whiteflies, but it differs somewhat from those associated with the aphid-transmitted carlaviruses (from Rodrigues et. al. 2014).

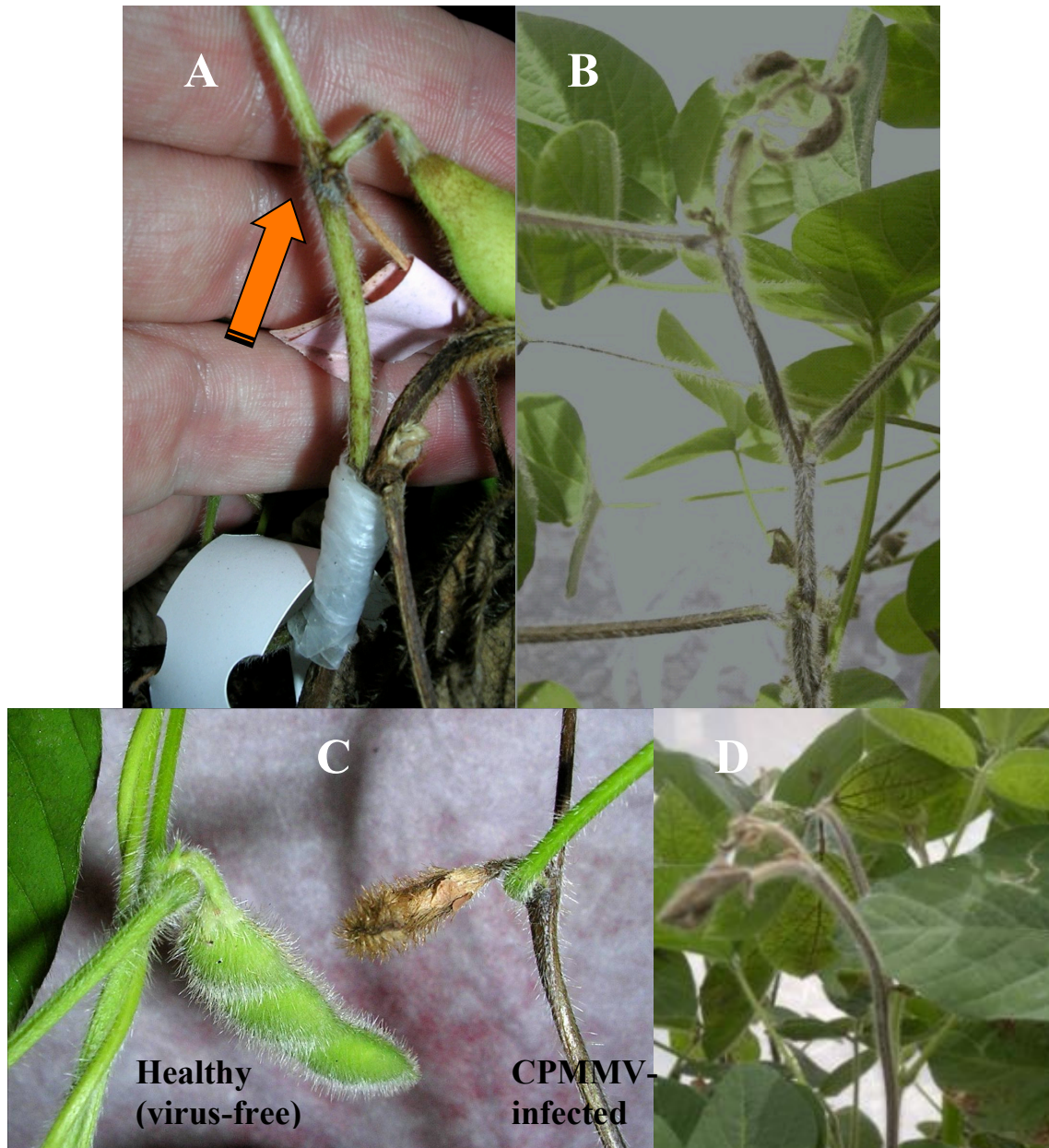




**Figure 3.** Field symptoms of *Cowpea mild mottle virus* (CPMMV) (A). Symptomatic pods and stem dieback (B). External symptoms in seeds collected from CPMMV-infected plants (top panel), and symptom-free seeds collected from healthy plants (lower panel).

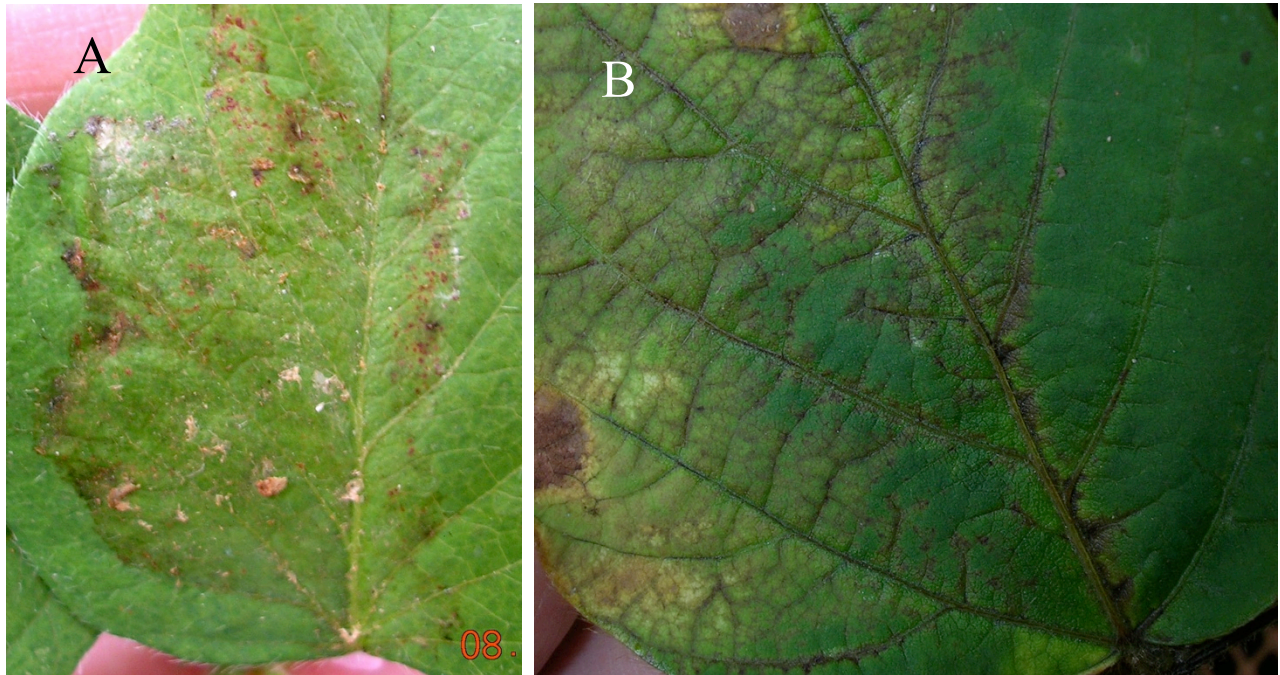


**Figure 4.** Transmission of CPMMV by graft-inoculation (A), whitefly transmission (B), and mechanical inoculation (C-D). Pods from mock-inoculated, healthy soybean plants (C), and pods from virus-infected, symptomatic soybean plants (D). Initial foliar symptoms developed 21 days post-inoculation, and symptoms were full-blown one week later in leaves, stems, and in pods, when present.





**Figure 5.** Mechanically-inoculated soybean leaves showing initial necrosis (A), and necrosis at the spreading edges of the site of inoculation (B).



**Figure 6.** Phylogenetic tree reconstructed of selected full-length genome sequences of *Cowpea mild mottle virus* and other carlaviruses, available in the GenBank database by Maximum Likelihood (1000 bootstrap iterations), showing a monophyletic species clade for CPMMV isolates, with two sister clades, each corresponding to a proposed strain.

